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TITLE: Does the Phenotyping of Disseminated Prostate Cancer Cells in Blood and Bone Marrow Prior to Radical Prostatectomy Provide Prognostic Information?

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ABSTRACT

Nearly 20% of men who undergo a radical prostatectomy (RP) later relapse with bone metastases. The cellular events that are predictive of subsequent progressive disease remain unknown. We've focused attention on the detection of disseminated prostate cancer (CaP) cells in the blood and bone marrow. Our hypothesis is that these disseminated cells may provide critical insight regarding biomarkers of use in prognostication. We've developed enrichment and isolation techniques that allow the isolation of individual disseminated CaP cells for study as a pool of cells or single cells. Our proposal was to isolate these cells from 50 patients prior to RP and from 10 patients with advanced disease. Once isolated, the cells are processed and stored for analysis. We greatly exceeded our accrual goal: 131 patients were accrued. We found that ~52% of patients prior to radical prostatectomy have disseminated CaP cells in their bone marrow. Molecular and immunohistochemical analyses are revealing the character of these cells. For example, 12% of the specimens show cells with Ki-67 proliferation staining. In addition, 65% of pre-RP specimens show chromosomal aberrations by FISH and aCGH, and RNA expression arrays suggest an epithelial to mesenchymal transition in the disseminated tumor cells.

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ADMINISTRATIVE NOTE

The vast majority of this report was submitted, reviewed and accepted by the DOD as the final report last year. However, since we obtained a no-cost extension to continue patient accrual and analysis, we have been requested to submit another report as the final. We have updated this report from last year as requested.

INTRODUCTION

One of the great challenges in the clinical management of prostate cancer (CaP) is determining the risk of progression in those patients thought to have localized disease. The recurrence rate among men who elect a radical prostatectomy for presumed localized disease ranges from 15 to 25%. Historically, two approaches have been taken to detect disseminated cancer cells and to improve the "staging" of patients at the time of diagnosis. In the first, microscopic scrutiny of disseminated cells in the blood of cancer patients by immunohistochemistry (IHC) and cytogenetic techniques revealed important information regarding the features of these disseminated cells in general (1-7). On a parallel path were efforts employing molecular technology. For example, thirteen years ago we (8) and Moreno (9) were the first to propose using the molecular technique of reverse-transcriptase-polymerase-chain-reaction (RT-PCR) to "molecularly stage" patients at diagnosis in hopes that detection of disseminated PSA+ cells in blood or bone marrow (BM) would be predictive of recurrence. Over time, efforts in this field showed that PSA RT-PCR positivity was not highly correlative with recurrence. However, we postulated that enrichment of the presumed CaP cells in the BM aspirate prior to RT-PCR testing might reveal a high pre-surgical detection rate and that techniques might then be developed to isolate the individual disseminated cells for study. We made use of magnetic particle cell enrichment techniques to study disseminated cells in CaP patients (10, 11, 16, 18). This approach provides for a much more robust analysis of the disseminated cell population, especially since the yield from BM aspirates can be several hundred to thousands of cells of interest. One of the most striking revelations in our series has been the documentation that ~50-55% of CaP patients prior to radical prostatectomy have disseminated cells in their BM aspirates following enrichment. The Objectives of this proposal center upon the study of these disseminated cells recovered from blood and BM and whether their biological features overall or as individual cells are predictors for progression. It appears logical that residual disseminated CaP cells in the BM (~90% of patients with advanced disease have bone metastases (12)) following a radical prostatectomy should provide at least as much insight as those associated with the primary tumor that is removed at surgery. The enriched population is adequate for microscopic analysis or for isolation of individual cells for further study. A powerful component of our proposed analyses involved cDNA micro-array-gene chips developed by our colleagues Leroy Hood and Peter Nelson. This technique combines the proven chemistry of nucleic acid hybridization with advanced automation and image analysis to quantitatively assess gene expression profiles (13-15). The expression levels of thousands to tens of thousands of genes represented at 0.01-0.001% abundance in a population can be simultaneously assessed. Thus our ongoing studies focus on generating molecular and IHC phenotypes of disseminated BM cells in fifty patients who are undergoing radical prostatectomy and having a Gleason Sum of 7 or greater. These profiles would then be contrasted to those of the primary tumor and to those in patients who have relapsed with bone metastases. A unique aspect of this proposal was our proposed profiling of individual disseminated tumors cells as the technologies advance to this level of sensitivity. Furthermore, comparisons of profiles would

have then be made between these patient populations and a subset that demonstrates PSA biochemical relapse. These studies were proposed to provide the first multiparameter phenotypic analysis/discovery of potential progression markers that takes advantage of our ongoing advances (a) in the recovery of disseminated CaP cells from blood and bone marrow, (b) in technology allowing the isolation of viable CaP cells to provide three degrees of heterogeneity (enriched, pooled homogeneous and individual cells) and (c) in the fields of cDNA microarrays and informatics that target small cell numbers and individual circulating tumor cells.

BODY

Hypothesis:

Disseminated CaP cells isolated from the blood and BM at the time of radical prostatectomy will reveal biological features useful in assessing the probability of relapse. Furthermore, the analysis of single cells isolated from the enriched population will provide a second level of discrimination reflective of the heterogeneity of disseminated cells and allow detection of rare, but important, features not revealed in pooled, groups of disseminated cells.

Technical Objectives:

Task #1: Define the predominant phenotype(s) of prostate cancer bone metastases

- Identify 10 patients with advanced prostate cancer involving the bone who have had a radical prostatectomy and were found to have a Gleason Sum $\geq$ 7. Following informed consent, obtain bone marrow aspirates. Using our para-magnetic enrichment techniques, we will derive an enriched population of disseminated CaP cells. From this population, we will "pluck" individual CaP cells and pool into sets of 50-100. These are then phenotyped using RT-PCR, PCR, FISH, ISH and micro-arrays (micro-array core facility of Peter Nelson, proposal co-investigator).
  (0 – 12 months)

Task #2: Establish phenotype(s) of primary tumor, and disseminated cells in blood and bone marrow by enrichment and pooling of cells from 50 patients undergoing prostatectomy and having Gleason Sum $\geq$7.

- Under separate funding we routinely obtain blood and bone marrow aspirates from all consenting patients prior to radical prostatetomy. From this large population this proposal involves a subset that consists of patients who are found to have a Gleason Sum $\geq$ 7. We will select 50 of these patients for the studies herein. Using our para-magnetic enrichment and isolation techniques, we will derive populations of disseminated CaP cells in three degrees of "purity": (a) enriched, (b) isolated individual cells that are pooled (50-100 cells) and (c) individual cells (20+). Using the phenotyping protocols of Task #1, sets (a) and (b) will be phenotyped from blood and from bone marrow. Set (c) will be partially purified and stored at –80° C for use in Task #3. We will also obtain at least 2 primary tumor foci (microdissected) for phenotyping.
  (0 – 24 months)

Task #3: **Establish the phenotype of individual disseminated cells following enrichment and isolation from the bone marrow in patients of Aim #2 who experience biochemical (PSA) failure.**

- Among the cohort of 50 patients, monitor for PSA biochemical recurrence using our ultrasensitive PSA chemiluminescent assay with which we've reported confidence in detecting relapse at a PSA serum level of 0.05 ng/mL. (0-30 months)
- Identify the first 10 patients who experience a biochemical recurrence and 10 who do not show any evidence of recurrence. Retrieve from –80°C storage the individual aliquots of disseminated CaP cells (bone marrow derived only) that were processed in Task #2. Phenotype, primarily by micro-array, these 400 individual CaP cells (20 cells/patient x 20 patients [10 fail + 10 no fail]). (20 – 36 months)

- Compare and contrast all data sets, perform statistical analyses. (30 – 36 months)

Results:

We were initially delayed by nine months in year 1, in our plan to accrue patients into the study because of a disagreement between the University of Washington IRB and the DoD over language in the consent form. A total of 60 patients were to be accrued, consisting of two patient populations, prior to radical prostatectomy and advanced disease. I am very pleased to reveal that we've far exceeded our accrual goals of 50 patients in the pre-radical prostatectomy patient group: 94 patients were accrued prior to the no-cost extension, and another 27 were accrued during this past year, bring the total to 121 patients. We were able to meet our accrual goal of 10 patients with documented advanced disease but only in the final months of our no-cost extension.

I mentioned in previous reports that with non-DoD funding, we acquired a better fluorescent microscope with which we achieved more accuracy in the detection of the disseminated cancer cells and the performance of fluorescent dependent phenotypic assays. I noted that there became a distinction among two populations of enriched epithelial cells from the bone marrow aspirates which had not been noticed with the older microscope. Our procedure uses negative (CD45 and CD61) selection followed by positive selection (anti-human epithelial antigen; HEA). The positively selected epithelial cells are detected under fluorescence using a FITC-labeled anti-HEA antibody to a different epitope than the antibody used for selection. The detected cells are then retrieved (i.e. "plucked") using a micromanipulator and pipette system attached to the inverted fluorescent microscope. In addition to the vast majority of non-fluorescent cells we noted now the two populations of fluorescent cells. One population was quite a bit more lightly stained than the other and in excess. The brightly stained population appeared to be the disseminated prostate cancer cells by morphology and molecular analysis whereas the lightly stained population appeared to be cells of other non-epithelial origin. An extensive literature search revealed one paper by Lammers et al (19) which stated that the human epithelial antigen resides in low abundance on a pre-erythroid stem cell population. Follow-up phone calls to other investigators using anti-HEA and to the commercial sources of anti-HEA

antibodies failed to derive supportive evidence of this work. However, because the selective "plucking" of these cells followed by RT-PCR failed frequently to reveal a PSA message, we decided to retrieve only the more brightly stained cells for study. The consequence of this decision was that far fewer cells were obtained than with the other older microscope which did not distinguish among the two populations. Because of this, we have been forced to reduce the number of descriptive phenotypic studies in some patient samples in preference to storing the most positive cells for micro-array analysis. This has especially impacted the peripheral blood specimens where we have preferentially stored the few positive cells per sample for future single cell micro-array studies rather than attempt phenotypic studies on just a very few cells. The percentage of patients prior to radical prostatectomy with disseminated cells in these 121 pre-radical prostatectomy patients is 34 % in the peripheral blood and ~52% in the bone marrow. Pools of positive cells and sets of individual positive cells have been obtained on all patients accrued to date where there were a sufficient number of disseminated cells detected. The radical prostatectomy patients were monitored for biochemical failure as part of Task 3. At a potential maximum of 40 months follow-up (actual median 25 months) post radical prostatectomy we've observed 17 biochemical recurrences among the 121 patients, 15 of which occurred during the no-cost extension year. Thus, we will begin retrieving and analyzing the disseminated cells from 10 of these patients and contrasting them to 10 who did not occur using, in part, the gene expression microarray techniques that now appear suitable for 10-cell aliquots (see below).

On all of the patients where we acquired a peripheral blood specimen and bone marrow aspirate prior to surgery we also obtained a tumor specimen at radical prostatectomy. These tumor specimens were embedded in OCT and flash frozen. As part of Task 2, two foci from each specimen in patients yielding a sufficient number of disseminated CaP cells for analysis, were to be microdissected for comparison to the results from the disseminated cells. We had proposed to do 50 tumor specimens but as noted above have now collected 121. Due to difficulty in scheduling sufficient time for microdissection on a core laser capture microdissection system, we acquired non-DoD funding to purchase a system for ourselves. With this new system we are about to complete the goal of 50 microdissections. As noted in the next paragraph, we with-held microarray analysis on these specimens until a reliable method was derived for the small cell number micro-arrays so that the identical method is used on both specimen types. During the no-cost extension, we appear to have achieved this goal and once verified, we will begin the analyses with non-DoD funding and will likely extend the number of tumor microdissections beyond 50 on a select patient basis (e.g. recurrences).

The ability to perform micro-array analysis on less than several hundred cells, let alone pools of approximately 10 cells or single cells as we proposed, has been an excruciating technical challenge to those in the field. We had hoped to report that this challenge has been resolved and that analysis of the stored disseminated cells described in Task 3 from our patients who had experienced a biochemical recurrence (N=10) and from those who had not (N=10) had been completed. While this is not yet the case, we are pleased that significant progress was made. Pete Nelson, M.D. who is our collaborator at the FHCRC and his team have worked persistently on this challenge. For example, we provided sets of LNCaP cells containing from 10 to 2500 cells to test the feasibility of two rounds of linear amplification followed by quantitative RT-PCR analysis. I am showing Figures 1 and 2 from previous reports again to highlight this issue. Up to 70 ug of aRNA was obtained from 10 LNCaP cells. Figures 1 and 2 demonstrate

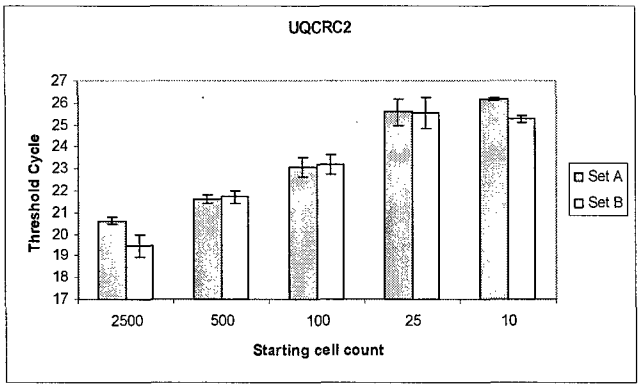

Figure 1

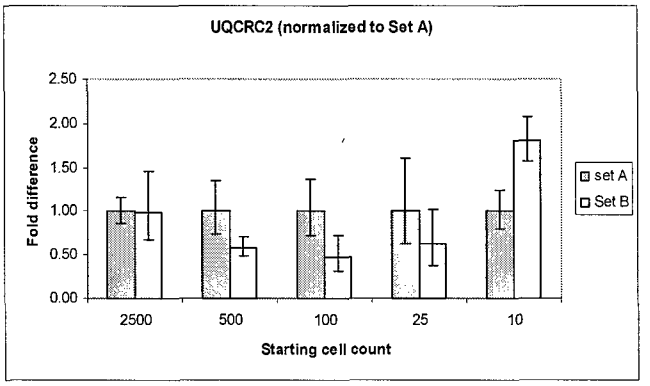

Figure 2

the success of these studies. In Figure 1, we demonstrate that the threshold cycle increases as the cell count decreases per unit aRNA reproducibly between sets; the difference between the samples with highest and lowest cell number is only 5-6 cycles. Figure 2 shows that throughout this range the fold difference is quite reproducible among samples, differing by less than 2 fold. I am very pleased that during the period of the no-cost extension, we were able to analyze a pilot set of 16 pre-radical prostatectomy and 4 advanced patients by quantitative RT-PCR for up to 7 targets, including cytokeratin, hepsin, PSA, and AMACR. Due to the success of this process, we continued efforts to fine-tune the gene-expression array procedure and have now completed expression analysis on 12 of these 20 specimens using 10-cell aliquots from patients with an excess of stored disseminated cells. Thus after nearly 4 years of effort, we believe we have achieved a method for getting down to the 10 cell limit with gene expression arrays and definitely with quantitative RT-PCR. Our goal remains to get down to the one cell limit and we will continue such efforts with non DoD funds. However, most important, we believe we are now very close to beginning the analysis by gene expression arrays of those stored disseminated cells from the patients who biochemically recur in contrast to those who do not (N=20 total). This work will be supported by discretionary funds received by my laboratory as soon as we are absolutely confident in the reproducibility of the 10-cell aliquot gene expression array analysis.

Progress has also been made in the phenotypic analysis of the disseminated cell population from the BM aspirates. Using a cytokeratin stain, we obtained percentages of patients positive for cytokeratin positivity (82%) and HEA positivity (57%) in BM. In the study of double-staining for cytokeratin and Ki-67, 12% (12/101) of the patients with cytokeratin positive cells also revealed Ki-67 stained cells. Within this small population, the percentage of cytokeratin positive cells that were Ki-67 positive was generally <10%. Thus, very few of the disseminated prostate cancer cells in the pre-radical prostatectomy population are actively undergoing division. A rare example of a Ki-67 positive cell (also cytokeratin positive) obtained

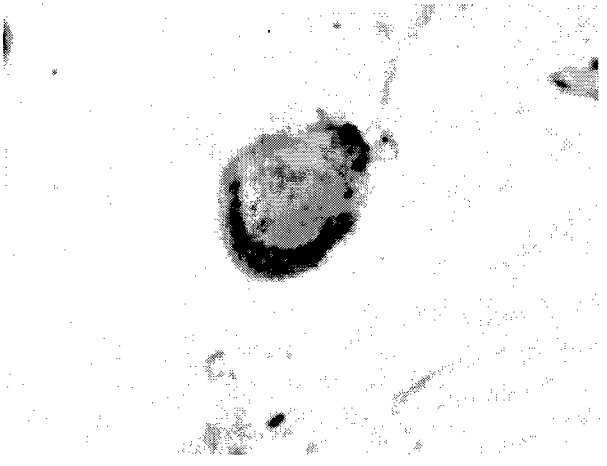

Figure 3

from a bone marrow aspirate is shown in the adjacent figure (shown in previous reports).

One interesting notation that conforms to our previous observations is that approximately a third of the patients with HEA positive and/or cytokeratin positive cells do not have PSA staining cells. This implies that these cells, once separated from the supportive stroma, stop producing PSA at levels necessary for immunohistochemistry detection. In fact, during the no-cost extension period, we evaluated a number of these cells by quantitative RT-PCR and found evidence of epithelial to mesenchyme transition (EMT). This is a very interesting observation as it suggests an interim phase where "some" of the circulating tumor cells undergo this EMT for a period of time perhaps lasting years during a period of dormancy. Then, by the time there is clinical evidence of micrometastases in the bone, at least some of these cells have converted back to phenotypic portrait of PSA expressing epithelial cells. Further follow-up investigation of these processes is warranted and if the data continue to support these initial findings, we intend to seek funding support for additional studies.

For RT-PCR analysis, we had originally planned on assessing Prostate Stem Cell Antigen (PSCA) and Prostate Specific Membrane Antigen (PSMA) on the enriched cell population but after dozens of analyses, concluded that there was too much background from the contaminating white blood cells to make an accurate determination of whether the signal was indeed from disseminated prostate cancer cells. Therefore, we attempted to do these analyses on ~10 – 20 of the individually "plucked" and then pooled cells from the enriched bone marrow aspirates. Using a pool of 10-20 LNCaP cells we can consistently obtain a positive PSMA RT-PCR result. We decided to convert to a real-time RT-PCR assay but our analysis of 10 cell aliquots was not reproducible. We are very cautious about using the actual 10-20 disseminated cell pools because they are irreplaceable. Therefore, this work was put on hold. In regard to PSCA, we are similarly not optimistic. Using a 10-20 cell pool we have been unable to consistently obtain the sensitivity and specificity required. Thus, at this point, we do not believe we will be able to include PSCA RT-PCR in the analysis. We previously discussed the possibility of using EZH2 (17) RT-PCR instead of PSCA RT-PCR. Here we encountered a different problem. Although we could get the sensitivity down to 10-20 cells routinely and one CaP cell frequently, we consistently obtained a positive EZH2 RT-PCR result on white blood cells. Thus, it is not a reliable specific marker for CaP cells even in the pooled disseminated cell population, since a few contaminating cells among the 20 "plucked" cells could give a false positive result. These difficulties highlight the significant technical hurdles being encountered in the accurate phenotypic and molecular assessment of individual and extremely small cell populations. Even with the no-cost extension, we have been unable to resolve some of the insurmountable technical hurdles. Nevertheless, we are fortunate to have discretionary funding that will allow some continued effort to solve these problems.

We greatly exceeded our goal of analyzing 50 bone marrow specimens by FISH. To date the majority of the 121 bone marrow specimens having cytokeratin positive cells, have been analyzed by FISH (N=70; Vysis ProVysion). This multiprobe kit for chromosome 8 allows for detection of increase or decrease of chromosome 8 and deletion/loss of 8p/8q. We had initially analyzed all specimens regardless of the number of cytokeratin positive cells (N=52 specimens). However, it soon became apparent that those with a low number of cytokeratin cells were not very reliable. Therefore, for final analysis we only evaluated those with 10 or more cytokeratin positive cells. The detailed analysis is underway but in 22 of 39 specimens (56%), chromosome 8 aberrations were noted. This is further evidence that malignant cells comprise at least a

segment of the disseminated cell population. Figure 4 shows a disseminated, cytokeratin positive cell (A) with 2 copies of chromosome 8 and 8q with one copy (red) of 8p (B).

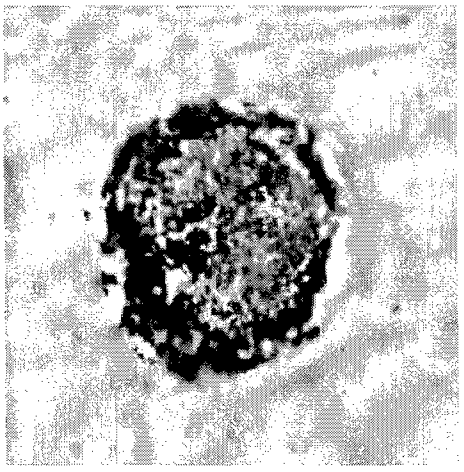

Figure 4A

Figure 4B

During the no-cost extension we began a pilot study to complement our FISH data with a more comprehensive assay, referred to as array comparative genomic hybridization (aCGH). In some respects similar in concept to gene expression arrays, this technology allows one to delineative chromosomal abnormalities across the genome in a single array format. However, also like the gene expression arrays, this technique typically utilizes thousands of cells as starting material for the DNA, not the 10 cell aliquots or single cell aliquots that we were proposing. With support from SPORE funding, our collaborator Barbara Trask, Ph.D. and her graduate student Ilona Holcomb had begun to enhance the sensitivity of aCGH. During the past year, we provided 10-cell aliquots of circulating tumor cells from 38 of our patients. To date, we've observed chromosomal gains/losses in 22/34 (65%) patients with localized disease prior to radical prostatectomy and 4/4 patients with advanced disease. Some of this work was presented at this year's annual American Urological Association meeting and is listed under the Reportable Outcomes section. These are very exciting data that demonstrate early genomic aberrations that occur in the disseminated tumor cell population in these prostate cancer patients. We will soon have matched gene expression array and aCGH datasets on the same patients.

KEY RESEARCH ACCOMPLISHMENTS

- Following a very slow start of patient accrual in year 1 due to IRB administrative issues, we have now exceeded our goal of 60 total patients by enrolling 121 pre-radical prostatectomy patients and 10 advanced disease patients. Due to low disseminated cell numbers in some of these pre-prostatectomy patients we increased the accrual target. We received a no-cost, one year extension to accrue additional pre-radical prostatectomy patients (N=24), to hopefully allow time for biochemical recurrences (only 2 had occurred at the time of last year's report) to occur in 10 patients and to devote additional effort in solving the technical hurdles in molecularly phenotyping individual and small sets (10 cells) of cells. As noted herein, significant success was noted in all three areas, including an additional 15 recurrences.

- We've made extensive progress in the phenotypic analysis of the disseminated cells from the bone marrow aspirates using the markers PSA, cytokeratin 18 and Ki-67. However, the proposed GSTpi studies have not given reproducible results at single cell and extremely low cell numbers.

- Good progress has been made in assessing specific gene expression profiles of isolated pools of 10 individual cells by RT-PCR or real-time RT-PCR including PSA, EGP, cytokeratin 18, AMACR and hepsin. AR methylation studies were begun only in those patients where sufficient disseminated cells are available as it is of lowest priority of the phenotypic studies. Our efforts to analyze PSCA on 10 or fewer cells reached an impasse, i.e. we could not reproducibly achieve the sensitivity and specificity required despite considerable effort. This again highlights the technical hurdles that were encountered as we pushed the limits of molecular phenotyping down to single cells or pools of 10 cells. With clinical specimens of individual and pooled cells so precious, we could not afford to engage in analysis of these specimens unless we were confident that the results were accurate.

- We were initially concerned that many of the disseminated tumor cells in the pre-radical prostatectomy patients did not express PSA by immunohistochemistry or by molecular analysis. However, during the no-cost extension year, evidence was generated that supported our hypothesis that this could be due to lack of stromal cell contact and an epithelial to mesenchymal transition. While these data are preliminary, they are very exciting and could explain in part the concept of tumor cell dormancy in these patients. Then, once the cells begin to establish themselves as micrometastases in bone, at least some of the disseminated tumor cells revert to their epithelial phenotype and again express the commonly associated prostate epithelial markers, such as PSA. This observation may serve as a new funding opportunity for the study of tumor cell dormancy.

- Four years of intensive efforts have finally achieved what appears to be successful amplification and suitable data from 10-cell aliquots by gene expression microarrays. These successes were only recently achieved during the no-cost extension year and validation is certainly required. As noted in the previous progress reports, pools of single disseminated cells and the individual cells have been "plucked" and stored from the patient samples but because they were so valuable, we didn't engage in microarray analysis until the procedure was found to be robust. This was a huge technical challenge, as were many aspects of this proposal. Credit is given to our collaborator Peter Nelson who was persistent in these efforts. While we have not yet mastered the technique required for single cells, efforts will continue. We intend to use non-DoD funding to begin the gene expression array analysis of selected aliquots (e.g. biochemical failure vs non-failure) of the stored 10-cell pools in hopes of generating sufficient data to apply for additional funding to expand the dataset beyond the original goals of this proposal.

- With non-DoD funding we acquired our own laser capture microdissection system. This has allowed a much more rapid dissection of tumor foci from the primary tumor removed at radical prostatectomy. In fact, within the next 30 days we will have finished the goal

of microdissecting 50 matched tumor specimens. Although there are sufficient cells in these dissections to do cDNA micro-arrays, it is critical that the identical procedure be used when comparing the microdissected tumor foci to the disseminated cells. Fortunately, as noted below, we appear to have finally mastered the method of amplification and analysis by micro-arrays of 10 cell aliquots of the disseminated cells. Once verified, we will begin to analyze our tumor foci dissections which are now being stored at -80° C using discretionary funding provided to my laboratory.

- Our genomic analyses by FISH were quite successful on the disseminated tumor cells showing that 56% of the patients had genomic aberrations in chromosome 8p/8q. During the no-cost extension period we complemented these studies with a pilot study of aCGH. As detailed herein, these studies are extremely exciting --- first in the development of an aCGH technique that is highly reproducible using only 10-cell aliquots and second, in the findings that 65% of the 34 patients prior to radical prostatectomy had demonstrable genomic aberrations. In the 4 advanced disease patients studied to date, all had genomic abnormalities in their disseminated tumor cell 10-cell aliquots. Continued funding of the aCGH work will be accomplished using SPORE funding resources.

- As we conclude this project, we intend to maintain the individual cell and 10-20 cell aliquots currently in -80° C storage from the 131 patients and to selectively retrieve them for analysis once the desired technologies are suitably robust. These are extremely valuable resources that will be utilized in the future and although DoD funding has now ceased, we will take advantage of our discretionary funding, SPORE funding and new funding opportunities to continue the study and analysis of these isolated disseminated tumor cells.

REPORTABLE OUTCOMES

Abstracts (cumulative):

Pfitzenmaier J, Arfman E, Klein J, Winch R, Nance M, Lange P and Vessella RL. New enrichment method for the isolation and characterization of circulating prostate cancer cells (CPCC) from the peripheral blood (PB). Proc. Am Assoc. Cancer Res 43:433 #3635, 2002

Pfitzenmaier J, Ellis WJ, Arfman E, Klein JR, Lange PH and Vessella RL. A method to isolate disseminated prostate epithelial cells and the comparison of their detection rate to standard RT-PCR. Proc. Am Assoc. Cancer Res 44:42, #216, 2003.

Pfitzenmaier J, Ellis WJ, Arfman EW, McLaughlin PO, Lange PH and Vessella RL. Telomerase activity in circulating prostate cancer cells. Proc. Am Assoc. Cancer Res 44:42, #5193, 2003

Lin DW, Pfitzenmaier J, Ellis WJ, Arfman EW, Klein JR, Lange PH and Vessella RL. Detection and isolation of PSA positive epithelial cells by enrichment: comparison to standard PSA RT-PCR, clinical relevance and initial characterization in prostate cancer patients. American Urological Association Annual Meeting, Abstract #837, 2004.

Holcomb, IN, Kinnunen M, Neal CL, Grove DI, Arfman EW, Lange PH, Hsu L, Delrow JJ, Lin DW, Trask BJ and Vessella RL. Array comparative genomic hybridization analysis of disseminated epithelial cells from the blood and bone marrow of men with clinically localized prostate cancer. American Urological Annual Meeting, 2005. J Urol 173(4): 61 #220, 2005.

Manuscripts:

Pfitzenmaier J, Ellis WJ, Hawley S, Arfman EW, Klein JR, Lange PH, and Vessella RL. The detection and isolation of viable PSA positive epithelial cells by enrichment: a comparison to standard PSA RT-PCR and its clinical relevance in prostate cancer. (under revision, 2005).

Chapters:

Lin DW, Pfitzenmaier J, Ellis WJ, Lange PH and Vessella RL. The detection, isolation and characterization of disseminated prostate cancer cells in the peripheral blood and bone marrow. Ed: Carrier Rinker Schaeffer (in press, 2005).

Tissue and Cell Banks:

We have collected matched pairs of tumor and non-malignant tissue from all 121 patients who underwent radical prostatectomy. From these patients and from the 10 with advanced disease we have prepared "enriched" pools of disseminated tumor cells suitable for immunohistology and isolated individual disseminated tumor cells in aliquots of 10-20 cells and individual single cells from all specimens where sufficient disseminated tumor cells were available. This is an invaluable resource for ongoing and future molecular studies of the disseminated tumor cell population from the time of diagnosis through quite advanced, metastatic disease.

Personnel Supported by these DoD funds:

Edward W Arfman
Martin Kinnunen
Jeff Klein
Bryce Lakely
Patrick McLaughlin
Barbara Ohara
Anita Simmons
Robert L Vessella
David Wallace
Rachel Winch

CONCLUSIONS

This is our final report and includes updates from our one year no-cost extension. Our overall patient accrual (N=131) is a great contrast to the slow start in year 1 due to the nine month delay in patient accrual due to DoD IRB issues. We exceeded our pre-radical prostatectomy goals by ~140% (121 vs 50) and met our goals for advanced stage patients (N=10). We believe the increased accrual was justified in that a number of patients did not yield a sufficient number of isolated disseminated prostate cancer cells to perform all of the studies proposed. We have processed all of the peripheral blood and bone marrow aspirates, enriching for disseminated prostate cancer cells. On those specimens with evidence of disseminated prostate cancer cells we have made suitable progress in both the phenotypic and molecular characterization of these cells, including finding that over 50% of the patients with disseminated tumor cells prior to radical prostatectomy and all with advanced disease had evidence of chromosomal aberrations among the disseminated tumor cells by FISH. Aliquots of individual disseminated cells have been "plucked" and stored in 10-cell aliquots and as 10 individual cell aliquots. From 10 patients who demonstrated a biochemical recurrence and from 10 who did not, we were to retrieve these cells and perform gene expression microarray analysis. While we awaited evidence of biochemical analysis we spent all three years of the regular funding period and much of the no-cost extension year in developing a reproducible gene expression array technique that could function well with just a 10-cell aliquot with hopes of getting the sensitivity down to single cells. Based on progress made during this past year, it appears we have achieved a suitable technique. The biochemical recurrences have occurred at a lower frequency than we anticipated so we began some initial studies on those patients where an excess of disseminated cells had been stored. These results provided the impetus for believing the process is now viable. In addition, during this no-cost extension the biochemical recurrences rose from 2 to 17 patients. We will now begin with non-DoD funding to analyze those stored 10-cell aliquots. Similar to the gene expression microarray studies, we began a collaboration to utilize aCGH as a genome-wide approach for analysis of genomic aberrations as a complement to the FISH studies. Here too, the challenge was to develop a method that would be reproducible with 10-cell aliquots rather than the 1000's of cells typically needed. During the non-extension year, this was solved and we demonstrated that 23/34 patients prior to radical prostatectomy presented with disseminated tumor cells with chromosomal abnormalities, i.e. gains/losses. To our knowledge, we still remain the only group applying the most advanced micro-array expression technologies and aCGH with those of classical phenotyping and FISH analysis to the study of disseminated prostate cancer cells isolated from the bone marrow down to the single cell level. While the technical challenges must not be underestimated to move this technology down to the single cell level and thus some goals may not be achieved because of this, the opportunities for advancement of knowledge remain highly attractive. The one year no-cost extension provided additional time to resolve some of these technical hurdles and now with non-DoD support, we are in a position to pursue the analysis on at least some of the more interesting subsets of the acquired and stored disseminated tumor cells.

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